Euphytica (2011) 181:77–88
DOI 10.1007/s10681-011-0422-1

Microsporogenesis of *Rps*8/*rps*8 heterozygous soybean lines

Maria Andrea Ortega · Anne E. Dorrance

Received: 14 November 2010 / Accepted: 25 March 2011 / Published online: 7 April 2011

Abstract Phytophthora root and stem rot caused by *Phytophthora sojae*, is one of the most damaging diseases of soybean, for which management is principally done by planting resistant cultivars with race specific resistance which are conferred by *Rps* (Resistance to *Phytophthora sojae*) genes. The *Rps*8 locus, identified in the South Korean landrace PI 399073, is located in a 2.23 Mbp region on soybean chromosome 13. In eight cv. Williams (*rps8/rps8)* × PI 399073 (*Rps*8/*Rps*8) populations, this region exhibited strong segregation distortion. In a cross between the South Korean lines PI 399073 (*Rps*8/*Rps*8) and PI 408211B (multiple *Rps* genes) this region segregated in a Mendelian fashion. In this study, microsporogenesis was evaluated to identify meiotic abnormalities that may be associated with the segregation distortion of the *Rps*8 region. Pollen was collected from greenhouse-grown plants of the parental genotypes: Williams, PI 399073, and PI 408211B; as well as selected *Rps*8/*rps*8 RILs from Williams × PI 399073 $BC_4F_{2:3}$ and PI 399073 × PI 408211B $F_{4:5}$ populations. There were no differences for pollen viability among the genotypes. However, for PI 399073, a mix of dyads, triads, tetrads and pentads was observed. A high frequency of meiotic abnormalities including fragments, laggards, multinucleated microspores; and microcytes containing DNA was also observed in *Rps*8/*rps*8 Williams × PI 399073 $BC_4F_{2:3}$ RILs. These meiotic abnormalities may contribute to the high degree of segregation distortion present in the Williams × PI 399073 populations.

Keywords Microsporogenesis · Segregation distortion · Soybean · Pollen · Resistance gene

Introduction

Glycine is a genus of leguminous plants, which includes the cultivated soybean (*Glycine max* [L.] Merr.), the wild annual soybean (*Glycine soja* Sieb. & Zucc.), as well as a number of perennial species (Kollipara et al. 1995; Hymowitz 2004). *Phytophthora sojae* Kaufm. & Gerd. is an oomycete pathogen of soybeans, causing root and stem rot in older plants, and damping-off of seedlings. Annual worldwide losses to Phytophthora root and stem rot can reach US $1–2 billion (Wrather et al. 2001; Wrather and Koenning 2006). The disease is managed through the deployment of single genes (*Rps* genes) that confer resistance to *P. sojae*. Currently fourteen *Rps* alleles have been reported at eight different loci. The *Rps*8 locus was identified in the South Korean landrace PI 399073 (Dorrance and Schmitthenner 2000), and was assigned to the soybean molecular linkage group

M. A. Ortega · A. E. Dorrance (✉)
Department of Plant Pathology, The Ohio State University, Wooster, OH 44691, USA
e-mail: dorrance.1@osu.edu

(MLG) F (Gordon et al. 2006) corresponding to chromosome 13 of *G. max*.

Multiple mapping populations were developed in recent years by crossing PI 399073 and the cultivar Williams, which is considered as the universal susceptible genotype to *P. sojae*. These segregating populations were advanced for several generations for the purpose of the identification of molecular markers for the development of breeding material carrying the *Rps*8 locus, and to assist in the cloning of the gene. Each population was phenotyped for resistance to *P. sojae* and genotyped with a set of markers on chromosome 13. The resistance phenotype was associated with markers in the *Rps*8 locus region (Ortega et al. 2010). However, the expected phenotypic and genotypic segregation ratios were always highly skewed (Ortega et al. 2008, 2010). In each of the populations derived from Williams × PI 399073 crosses, an excess of *Rps*8/*Rps*8 homozygous RILs and *Rps*8/*rps*8 heterozygous RILs were obtained at the expense of the *rps*8/*rps*8 homozygous RILs.

Segregation distortion is not uncommon in mapping populations, and is one of the several factors that influence the precision of genetic mapping. It has been shown that both the genetic distance between markers and the order of the markers on linkage groups could be affected by this phenomenon (Lorieux et al. 1995a, b). In soybean, 18 regions on ten different linkage groups where high levels of segregation distortion occur were previously described (Yamanaka et al. 2001), including the soybean root fluorescence locus *Fr*1 on chromosome 9 (MLG K) (Jin et al. 1999). The mechanisms involved in segregation distortion are not well understood. However gametophytic factors, competition among gametes, or the abortion of gametes have all been proposed (Lu et al. 2000, 2002; Lyttle 1991; Matsushita et al. 2003).

Continued development of virulent pathotypes of *P. sojae* that can infect plants with *Rps* resistance genes (Grau et al. 2004) and the limited number of effective *Rps* genes that are currently deployed in US cultivars, makes the quest for novel sources of resistance a high priority. Sources of resistance to host-specific plant pathogens are usually found in the regions of greatest differentiation of host species (Leppik 1970). In the case of Phytophthora root and stem rot, sources of resistance have been identified in both Chinese and South Korean germplasm (Dorrance and Schmitthenner 2000; Kyle et al. 1998; Lohnes et al. 1996). However, phenomena like segregation distortion can complicate the development of resistant cultivars when genes are introgressed from novel sources of resistance. In the case of *Rps*8, segregation distortion in populations derived from PI 399073 represents a major challenge to fine map and clone this gene. Identifying the mechanisms that contribute to the segregation distortion of *Rps*8 is crucial for the identification of breeding strategies that will expedite the use of this gene for the management of Phytophthora root and stem rot of soybeans.

In plant development, microsporogenesis is the cellular division that produces haploid microspores which develop into pollen grains, and this is also an important process where meiotic abnormalities may be detected. A large number of microspores are produced in each anther, making them a feasible target for the evaluation of the different meiotic phases. Normal microsporogenesis in soybean has been described (Albertsen and Palmer 1979), and serves as a useful guide for the evaluation of abnormalities. Structural changes in chromosomes that effect the production of normal gametes, such as inversions and translocations (Mahama et al. 1999; Palmer et al. 2000) have also been previously described in soybean.

To produce pollen grains, male gametogenesis starts with the division of a diploid sporophyte that gives rise to both the tapetum and pollen mother cells (PMCs) (McCormick 1993). The later cells undergo meiosis and give rise to tetrad cells that are released as microspores when the callose is degraded by the enzyme callase produced by the tapetum. The microspores undergo mitosis to generate pollen grains containing the larger vegetative cell and the small generative cell. The irregularities reported in soybean male meiosis include: chromosome associations, abnormal spindles, precocious chromosome migration, chromosome stickiness, chromosome fragments, laggards, bridges, micronuclei, cytokinesis failure, and production of microcytes (Bione et al. 2000, 2002, 2005; Kumar and Rai 2006; Palmer et al. 2000). In general, abnormal male meiosis tends to have an outcome of partial or total pollen sterility. This is especially important in soybean, as this genus self fertilizes and genotypic factors associated with

those pollen grains would not be passed to the next generation.

Due to the high level of segregation distortion across the numerous crosses of PI399073 and *G. max* cultivars, a comparative study of *Rps*8/*rps*8 lines from two parental combinations, Williams × PI 399073 and PI 399073 × PI 408211B was initiated. Our objective was to determine if male gametogenesis abnormalities occur in *Rps*8/*rps*8 lines which originated from populations with a high degree of segregation distortion as well as examine populations where this locus segregates in a true Mendelian fashion. For each genotype, the targets were meiosis I and II, microspores, and mature pollen grains.

Materials and methods

Plant material

A $BC_4F_{2:3}$ population consisting of 30 lines was generated by backcrossing the region containing the *Rps*8 locus from PI 399073 into cultivar Williams (recurrent parent). BC_4 seeds were advanced by single-seed-descent to generate $F_{2:3}$ seeds. In a previous study, the population was phenotyped for resistance to *P. sojae* through hypocotyl inoculation. *P. sojae* isolate OH25, virulent to plants carrying the *Rps* genes 1a, 1b, 1c, 1k, and 7, was used to identify lines carrying the resistance locus *Rps*8 from PI 399073 in the $BC_4F_{2:3}$. In addition, a $F_{4:5}$ population consisting of 152 recombinant inbred lines (RILs) was generated by crossing PI 399073 and PI 408211B, F_2 seeds were advanced by single-seed-descent. The phenotypic data for disease resistance was obtained by inoculation with *P. sojae* isolate BUTMU. This isolate has a compatible interaction (susceptible response) with the *Rps* genes in PI 408211B, and an incompatible interaction (resistance response) in PI 399073. Lines with a heterozygous phenotype were genotyped with 72 SSR and SNP markers in the *Rps*8 region, and four lines were selected from each population for this study, these lines were heterozygous for the resistance phenotype and molecular markers located between the SSRs Satt114 and Satt362 (Table 1).

Seeds from each heterozygous line and parental genotype were planted in 2-l pots of sterilized soil mixture. Based on earlier genotypic data, recombinant inbred lines (RILs) 1403, 1404, 1412, and 1413 from the $BC_4F_{2:3}$; and RILs 37, 50, 58, and 143 from the $F_{4:5}$ populations were selected for this

Table 1 Genotype of the *Rps*8 region on the selected $BC_4F_{2:3}$ lines and $F_{4:5}$ RILs

Chromosome 13		Williams × PI 399073 $BC_4F_{2:3}$									PI 399073 × PI 408211B $F_{4:5}$							
		*Rps*8/*rps*8 lines									*Rps*8/*rps*8 lines							
		1403		1404		1412				1413	37		50		58	143		
Position Mbp[a]	Marker	1	2	1	2	1	2	3	4	1	1	2	1	2	1	1	2	3
27.71	Satt114	A	A	A	A	A	A	A	A	A	C	C	H	H	H	H	H	H
28.21	F420_18	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
28.41	Satt334	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
28.84	F336_18	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
29.01	98FA16.2	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
29.03	F336_01	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
29.27	F396_08	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
29.30	AC15916.2	H	H	H	H	H	H	H	H	H	H	H	H	C	H	H	H	H
32.86	Satt362	A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H

These plants are the progeny of *Rps*8/*rps*8 plants, and the maintenance of heterozygosity was confirmed on genomic DNA isolated from the seedlings. Williams homozygous locus (*A*), Williams/PI 399073 heterozygous locus (*H*), PI 408211B homozygous locus (*C*), and PI 408211B/PI 399073 heterozygous locus

[a] Position based on Williams82 8X assembly (Mbp) (available at http://www.phytozome.net/soybean.php)

experiment (Ortega et al. 2010). Plants were grown at 27°C with 14 h daylight period (with supplemental lighting provided); watered twice every day, and supplemented with 100 ppm 20N:20P:20K greenhouse fertilizer. Studies were from January to March 2009.

DNA isolation and genotyping

Genomic DNA was extracted using a modification of the protocol described by Keim et al. (1988). Cotyledons from 3 week-old seedlings were collected in 10 × 10 cm^2 reclosable plastic bags (Uline, Inc., Philadelphia, PA) and stored at 4°C until processing. Tissue was ground in CTAB buffer: 100 mM Tris–HCl pH 8.0, 1.4 mM NaCl, 2.0% CTAB (hexadecyltrimethyl-ammonium bromide), and 20 mM EDTA pH 8.0. One milliliter of the extraction buffer was added to the plastic bag, and the tissue was macerated using a hand-held roller (BIO-RAD, Hercules, CA). The suspension was transferred to a 2.0 ml tube and incubated at 65°C for 1 h, and mixed vigorously every 15 min. The sample was cooled to 27°C, and an equal volume of 24:1 (v/v) chloroform-isoamyl alcohol (Sigma Chemical Co., St. Louis, MO) was added. An emulsion formed after inverting the tubes several times, followed by centrifugation at 10,000 rpm for 10 min in a table-top microcentrifuge. The supernatant was transferred to a 2.0 ml tube, and the DNA was precipitated from the solution by adding 99% isopropyl alcohol and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the DNA pellet was washed with 70% ethyl alcohol. The DNA pellet was air dried overnight and resuspended in 500 µl of TE buffer (10 mM, 1 mM EDTA pH 8.0). RNA was removed by treatment with 2.0 µl of 5 mg/ml Ribonuclease A (Sigma–Aldrich, St. Louis, MO) were added to the reactions and incubated at 37°C for 1 h. DNA was quantified by spectrophotometry using NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer instructions, and the samples were stored at −20°C.

To verify that each RIL carried heterozygous genotypes, each line was screened with eight SSR markers across the *Rps*8 region. The markers Satt114, Satt334, Satt362 located on chromosome 13 (MLG F) on the soybean consensus map (Cregan et al. 1999); AC15916, 98FA16; and F420_18, F336-01, and F336_18 developed from sequences from Williams82 BAC clones and mapped to chromosome 13 were used for genotyping. Template DNA was diluted to 50 ng/µl in TE buffer and stored at −20°C. The PCR amplification was done in a 12.5 µl reaction mixture containing 1× Green Go Taq Flexi Buffer (Promega, Madison, WI), 2 mM $MgCl_2$ (Promega), 200 mM of each deoxynucletide (Promega), 200 nM of each primer, 1 U Go Taq DNA polymerase (Promega), and 50 ng of genomic DNA. All PCR reactions were carried out on a DNA Engine Tetrad 2 Peltier Thermal Cycler (BioRad, Hercules, CA). The thermal conditions were 94°C for 5 min; ten cycles of touchdown PCR: 94°C for 45 s, 60–50°C (decreasing 1°C per cycle) for 45 s, and 72°C for 1 min; followed by 24 cycles with annealing temperature of 50°C; and final extension at 72°C for 10 min. PCR products were analyzed on 4.0% agarose 3:1 HRB (Amresco, Solon, OH). Agarose was dissolved in 1× RapidRun agarose buffer (USB, Cleveland, Ohio), pre-stained with 0.5 µg/ml of ethidium bromide (Sigma–Aldrich, St. Louis, MO) and cast in 20 × 25 cm trays (Fisher Scientific, Pittsburgh, PA). Ten microliter amplicons were electrophoresed in 1× RapidRun agarose buffer for 25 min at 250 V. Electrophoresed gels were visualized and digitally photographed.

Pollen viability and germination

Open flowers were collected between 9:00 and 11:00 a.m. during the first 3 weeks of flowering. Three flowers were collected from each plant, three times a week. Each set of anthers was dissected and dusted onto pollen germination medium (Gwata et al. 2003) and incubated at 27°C for 18 h. A minimum of 100 pollen grains per anther were observed for germination under a S6D Stereozoom microscope (Leica Microsystems Inc., Deerfield, Illinois, USA). A grain was classified as germinated if a recognizable pollen tube, at least 20 µm long was present. Pollen viability was assessed with Lugol's solution (Electron Microscopy Sciences, Hatfield, PA), consisting of 5% iodine and 10% potassium iodide, and this staining detects starch content. The same set of anthers used to determine percent germination were placed in 100 µl of Lugol's solution on a 25.4 × 76.2 mm slide (Becton–Dickinson Labware, Franklin Lakes, NJ). The slide was covered with a 22 × 40 mm cover glass (Daigger, Vernon Hills, IL)

and visualized under a binocular DME light microscope with the 20× magnification objective (Leica Microsystems Inc., Deerfield, IL). Pollen grains which were stained dark brown to black were considered viable.

Cytological analysis of male meiosis

In this study acetic carmine staining was used for visualization of the chromosomes (Schreiber 1954). Immature flower bud clusters were collected between 9:00 and 12:00 a.m., the stage reported for meiotic analysis in soybeans (Bione et al. 2003; Mahama et al. 1999; Palmer et al. 2000). Flower buds from single plants were placed in 2.0 ml microcentrifuge tubes containing 1.5 ml of formalin-aceto-alcohol mixture (Ricca Chemical Company, Arlington, TX) for fixation. The samples were incubated at 27°C for 24 h and stored at 4°C until assayed.

Anthers were dissected and transferred to 0.2 ml tubes containing 0.75% acetic carmine (Carolina Biological Supply Company, Burlington, NC). Carmine was dissolved in 45% acetic acid, and it served the double purpose of fixation and staining; acetic acid penetrates membranes rapidly, and carmine is insoluble in chromatin. The staining was enhanced by adding 2 µl of 10% w/v ferric chloride solution (Sigma Chemical Co., St. Louis, MO). Dissected anthers were incubated at 70°C for 8 h and maintained at 27°C for another 24 h. The anthers were blotted on Kimwipes (Kimberly-Clarke, Roswell, GA) and placed on a 25.4 × 76.2 mm slide (Becton–Dickinson Labware, Franklin Lakes, NJ) containing 100 µl of mounting media (Rattenbury 1956). The slide was covered with a 22 × 40 mm cover glass (Daigger, Vernon Hills, IL). The slides were placed on the dissecting scope and each anther was crushed, by applying pressure on the cover glass with a dissecting needle, until the anther wall broke and meiotic cells were released. The preparations were sealed with nail polish.

The preparations were viewed under a binocular DME light microscope (Leica Microsystems Inc., Deerfield, Illinois, USA) at 1000× magnification, and photographed using a Nikon digital sight DS-SM camera and DS-L1 computer (Nikon Corp., Japan).

Results

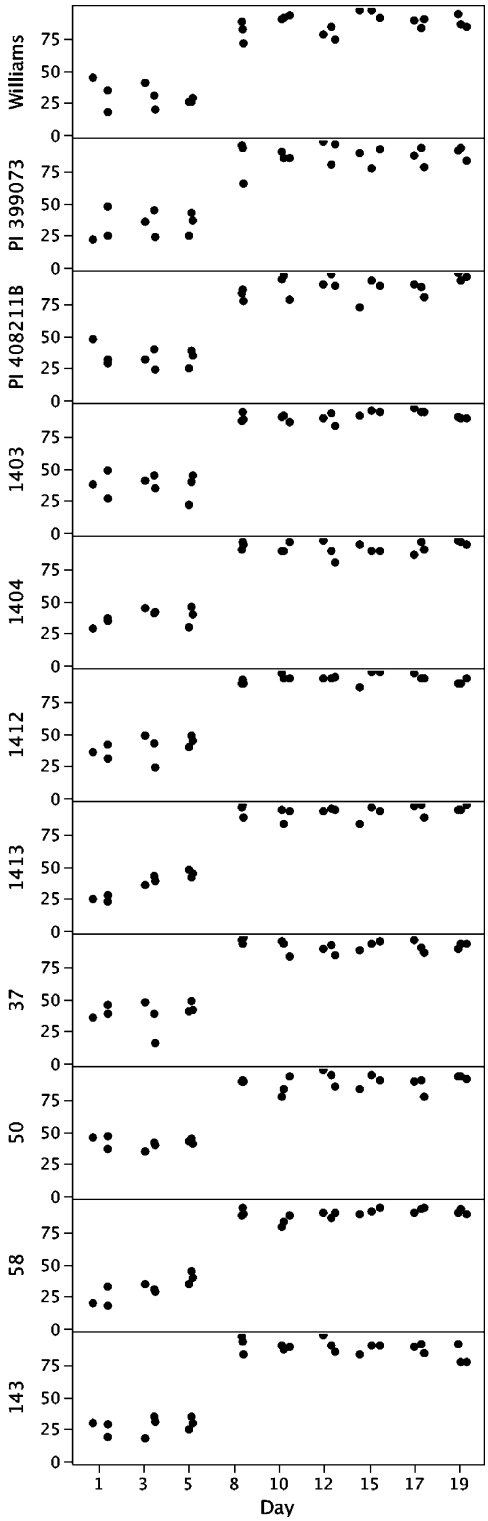
Pollen viability

One plant from each line and parents was used for evaluation of pollen viability, this was done so that it would be possible detect variation for this parameter between the different collection times, and at the same time leave enough immature flowers for the cytogenetic studies. A mean of 90% of the pollen grains stained dark brown with Lugol's solution indicating viability (data not shown). In addition, there was no significant difference among plants for pollen germination on the same sampling day, but a significant ($P = 0.05$) difference was found between sampling days for the same plant (Fig. 1). Pollen collected from flowers produced on the first week of the reproductive stage, independently of the plant evaluated, had germination percentages lower than 55%. The percentage of pollen grains that germinated increased in the second week, and was maintained above 85% during the third week of flowering.

Cytogenetics

During the microsporogenesis process in PI 399073 and RILs from the $BC_4F_{2:3}$ Williams × PI399073 several meiotic abnormalities were observed from the stained anthers collected from immature flower clusters. Pollen abnormalities were not found in PI 408211B nor in Williams. For the Williams × PI 399073 $BC_4F_{2:3}$ derived plants, there were 255 abnormal meiotic cells from the 371 meiotic cells evaluated; this number was higher than the observed in any other genotype. For the PI 399073 × PI 408211B $F_{4:5}$ lines, only 9 of 396 cells exhibited abnormalities (Table 2). During meiosis I, the following abnormalities were observed: extra nucleolus (Fig. 2a) in the pollen mother cells at prophase I, chromosome fragments that were not part of the metaphase plate (Fig. 2b–d), laggards present between the two chromosomes sets at anaphase I, and micronuclei formed between the two nuclei at telophase I (Fig. 2f). In the fixed flower buds of the genotypes, anthers at meiosis I were identified more frequently than anthers at meiosis II. Similar meiotic abnormalities were also observed in meiosis II.

The types of abnormalities of the male gametes were determined by observation of the microspores



◄ **Fig. 1** Pollen germination percentage for one plant from each line which was evaluated from the beginning of the reproductive phase (R1). Three open flowers were collected 3 days per week for 3 week. The percentage of germinated grains was determined after 18-h incubation in the medium described by Gwata et al. (2003)

formed after meiosis II (Table 2). Flowers in the same cluster were each in a different stage of microsporogenesis, thus the characteristics of microspores and pollen grains were noted for each genotype (Table 3). In PI 399073, a mix of dyads, triads, and pentads were found (Fig. 3). In this parental genotype, tetrads comprised only 67% of the meiotic products; and 63% of them had micronuclei in at least one of the microspores. For Williams and PI 408211B, only tetrads were observed. In selected lines from the Williams × PI 399073 $BC_4F_{2:3}$, microspores containing micronuclei were common (Fig. 2j, k), and 'triads' containing two microspores and a microcyte were also observed (Fig. 2i). This type of triad was only observed in the anthers from the *Rps*8/*rps*8 Williams × PI 399073 $BC_4F_{2:3}$ RILs. Uninoculated microspores with thick cell walls were formed in the non dehiscent anthers from PI 399073, PI 408211B, the *Rps*8/*rps*8 Williams × PI 399073 $BC_4F_{2:3}$ RILs, and the *Rps*8/*rps*8 PI 399073 × PI 408211B $F_{4:5}$ RILs (Fig. 4a, b, e, f). In contrast, in Williams, the majority of the microspores were in the binucleate stage, after mitosis I (Fig. 4c); germinated pollen grains were also common inside the non dehiscent anthers of cultivar Williams (Fig. 4d). A few non-viable pollen grains, not stained with acetic carmine, were observed in the immature anthers of all the genotypes evaluated (Fig. 4b). Pollen grains from the anthers of *Rps*8/*rps*8 $BC_4F_{2:3}$ RILs were one-third the size of an average pollen grain and contained one or more micronuclei, the cytoplasm in these grains was not darkly stained but a cell wall like structure was observed (Fig. 4e). These small grains corresponded to 87% of the sterile pollen found in the *Rps*8/*rps*8 $BC_4F_{2:3}$ RILs, and may be the product of the microcytes formed in earlier stages. However, these grains were not detected when dehiscent anthers were used for evaluation of pollen viability and germination, indicating that these small grains may collapse and degrade before anthesis.

Table 2 Pollen meiotic abnormalities in PI 399073, PI 408211B, Williams, and selected *Rps*8/*rps*8 heterozygous RILs from: Williams × PI 399073 $BC_4F_{2:3}$, and PI 399073 × PI 408211B $F_{4:5}$ crosses

Phase	Abnormalities	Number of cells analyzed and cells exhibiting abnormalities																					
		Parental genotypes						Williams × PI 399073 $BC_4F_{2:3}$ *Rps*8/*rps*8 lines								PI 399073 × PI 408211B $F_{4:5}$ *Rps*8/*rps*8 lines							
		PI 399073		PI 408211B		Williams		1403		1404		1412		1413		37		50		58		143	
		No.[a]	A[b]	No.	A	No.	A	No.	A	No.	A	No.	A	No.	A	No.	A	No.	A	No.	A	No.	A
Meiosis I																							
Prophase I	Extra-nucleoli[c]	33	2	110	0	28	0	12	0	8	1	20	1	44	0	21	0	7	1	8	0	3	0
Metaphase I	Fragments[d]	29	2	12	0	69	0	18	14	10	10	22	18	6	5	8	0	42	1	28	1	6	0
Anaphase I	Laggards[e]	36	3	7	0	14	0	36	34	17	17	23	22	8	8	31	0	19	2	24	0	34	1
Telophase I	Micronuclei[f]	N[g]	–	15	0	23	0	30	29	19	17	20	18	9	9	35	0	28	1	13	1	30	1
Meiosis II																							
Metaphase II	Fragments	6	0	N	0	13	0	8	6	5	4	6	5	6	6	6	0	5	0	9	0	5	0
Anaphase II	Laggards	N	–	3	0	5	0	N	0	2	1	4	4	4	2	N	0	3	0	N	–	2	0
Telophase II	Micronuclei	16	9	11	0	14	0	12	7	13	10	9	7	N	–	9	0	6	0	10	0	4	0

Anthers from the flower clusters were collected during the first 3 weeks of flowering and were stained with acetic carmine

[a] Total pollen mother cells evaluated (No.)

[b] Abnormal pollen mother cells (A)

[c] Darkly stained, nuclei buds on late prophase

[d] Precocious chromosome migration to the poles

[e] Chromosome lagging between the anaphase spindles

[f] Condensation of the lagging chromosomes

[g] N indicates that cells in this stage were not observed for this genotype

The meiotic abnormalities and meiotic products observed for the $BC_4F_{2:3}$ *Rps*8/*rps*8 heterozygous lines were also identified on the anthers of $BC_4F_{2:3}$ *Rps*8/*rps*8 heterozygous plants that were grown in a preliminary study, during the summer of 2008 (June–August). The plants evaluated on the preliminary study included four lines from the same $BC_4F_{2:3}$ population evaluated on this study, including RIL 1412, and six lines from another $BC_4F_{2:3}$.

Discussion

In this study the male gametogenesis in *Rps*8/*rps*8 heterozygous lines and their parents was evaluated. When pollen from the same anthers was studied in selected heterozygous RILs and parental genotypes in the first week of flowering, most of the grains stained with Lugol's solution, indicated that they were viable. However, a low percentage of pollen germinated under the test conditions across all genotypes. The cause of poor pollen germination during the first week of flowering in this study is unknown. Previously in soybeans, temperature, UV radiation, and CO_2 levels have been shown to affect pollen morphology and germination (Koti et al. 2005). Cytogenetic staining techniques were effective for the visualization of meiotic chromosomes and detection of abnormalities during their separation during haploidization. The products of male gametogenesis

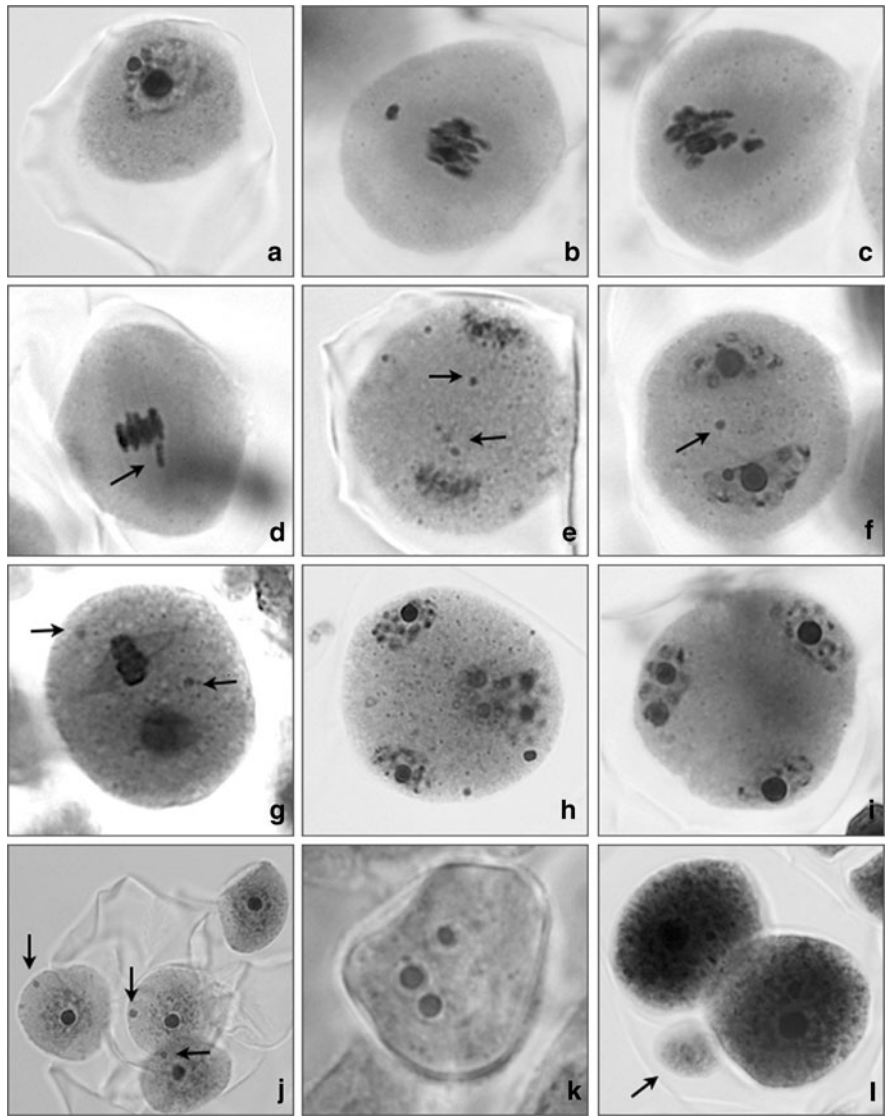


Fig. 2 Meiotic irregularities observed in Williams × PI 399073 $BC_4F_{2:3}$ Lines. **a** Prophase I, micronucleus. **b**, **c** Metaphase I, chromosome fragments. **d** Metaphase I, chromosome not aligned at the metaphase plate. **e** Late anaphase I, laggards. **f** Telophase I, micronucleous. **g** Metaphase II, Chromosome fragments. h, **i** Coenocytic tetrad, binucleate cells. **j** Tetrad cells, micronuclei. **k** multinucleate microspore. **l** Dyad, microcyte

were also subjected to analysis, and two methods were employed to determine the percentage of viable pollen in each genotype. The frequency of meiotic abnormalities was higher in *Rps*8/*rps*8 heterozygous lines from a Williams × PI 399073 $BC_4F_{2:3}$ population with a high degree of segregation distortion in the *Rps*8 region, than in *Rps*8/*rps*8 heterozygous lines from a PI 399073 × PI 408211B $F_{4:5}$ population in which the *Rps*8 region segregated normally in a Mendelian fashion.

Chromosome elimination affects the correct separation of chromosomes during cellular division and has been attributed to: chromosome fragmentation, micronucleous formation and chromatin degradation (Subrahmanyam and Kasha 1973; Thomas 1988); lagging chromosomes (laggards), bridges, chromosomes non-congregated at the metaphase plate, and failure of chromosome migration to the poles during anaphase (Bennett et al. 1976). Chromosome elimination has also been reported during microsporogenesis (Adamowski et al. 1998). In our study, the meiotic abnormalities observed during microsporogenesis, accompanied by the presence of microspores and microcytes containing micronuclei, indicates that chromatin elimination may be a potential mechanism influencing segregation distortion in these lines. This

Table 3 Characteristics of the meiotic products and pollen grains from PI 399073, PI 408211B, Williams, and selected *Rps*8/*rps*8 heterozygous RILs from: Williams × PI 399073 $BC_4F_{2:3}$, and PI 399073 × PI 408211B $F_{4:5}$ crosses

Male gametogenesis products	Parental Genotypes			Williams × PI 399073 $BC_4F_{2:3}$				PI 399073 × PI 408211B $F_{4:5}$			
				*Rps*8/*rps*8 lines				*Rps*8/*rps*8 lines			
	PI 399073	PI 408211B	Williams	1403	1404	1412	1413	37	50	58	143
Meiotic products[a]											
Dyads[b]	27	0	0	18	9	12	0	0	0	0	0
Multinucleated[c]	27	0	0	0	0	0	0	0	0	0	0
Triads[d]	22	0	0	0	0	0	0	0	0	0	0
Multinucleated	22	0	0	0	0	0	0	0	0	0	0
Tetrads[e]	137	149	81	61	86	95	69	10	84	77	111
Multinucleated	87	8	2	49	67	78	45	3	0	0	7
Pentads[f]	16	0	0	0	0	0	0	0	0	0	0
Multinucleated	2	0	0	0	0	0	0	0	0	0	0
Pollen											
Uninucleated[g] grains	312	301	25	199	216	204	141	220	250	297	298
Binucleated[h] grains	5	31	256	2	12	8	3	8	6	9	13
Sterile grains	8	4	4	5	0	8	6	7	3	1	4
Microcytes[i]	0	0	0	25	18	30	47	0	0	0	0

Type of meiotic products and the number of nuclei per cell were recorded for each genotype. The viability of the mature pollen was determined by the presence of acetic carmine in the cytoplasm

[a] Set of microspores, classified according to the number of microspores in each set

[b] Two microspores

[c] Number of sets in which at least one microspore contained more than one nucleus

[d] Three microspores

[e] Four microspores

[f] Five microspores

[g] Pre-mitotic microspores

[h] Microspore containing the generative cell

[i] Non-viable, small (<10 μm) meiotic products grains containing micronuclei

mechanism does not seem to have noticeable effects on pollen viability, because there was no correlation between meiotic abnormalities and the percentage of stained pollen or germinated pollen at later flowering dates. However there was a correlation between the frequency of meiotic abnormalities and the presence of microcytes. In particular, RILs from the Williams × PI 399073 $BC_4F_{2:3}$ where a high level of abnormalities were detected, the fertility of the mature pollen was not affected, indicating that the loss or gain of the micronuclei may not have a serious effect on the pollen grain viability.

Many mechanisms of chromosome elimination have been described (Singh 1993), however the process involved in the elimination of micronuclei as microcytes is still obscure. The elimination of micronuclei from microspores in oat (*Avena sativa* L.) was reported by Baptista-Giacomelli et al. (2000). A micronucleus reaches the microspore wall and separate from it by forming a bud, then the formed microcyte give rise to a sterile pollen grain; this process has not been described in any other species to date. Partial genome elimination through micronuclei and the production of aneuploid gametes was described in plants from a natural population of *G. max* (Kumar and Rai 2006). In this study tetrads containing quiescent micronuclei were also present, and pollen viability was not affected. The process that

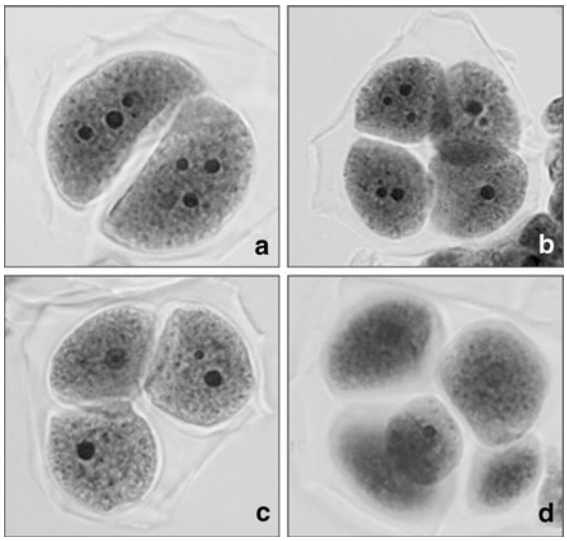


Fig. 3 Meiotic products in PI 399073: **a** multinucleate dyad, **b** multinucleate tetrad, **c** multinucleated triad, **d** mononucleated pentad

gave rise to the microcytes in the Williams × PI 399073 $BC_4F_{2:3}$ is not clear, although a similar mechanism is suspected since micronuclei in the tetrads were located close to the wall (Fig. 3j), and it is unlikely that the cell wall in the small pollen grains could have originated using the limited genetic material inside them. The maintenance of micronuclei within the microspores could be the result of low efficiency in the elimination process.

The identification of different types of meiotic products in PI 399073 and RILs of the Williams × PI 399073 $BC_4F_{2:3}$ was striking. Although these abnormal dyads, triads, tetrad, and pentads do not appear to have an effect on pollen viability, this phenomenon may not be uncommon as similar types of meiotic products have been observed in other species including a pentaploid accession of *Brachiaria brizantha* (Risso-Pascotto et al. 2003). In the microsporogenesis stage, micronuclei were formed and some remained inside the microspores, while others were eliminated as microcytes in a similar mechanism to the described by Baptista-Giacomelli et al. (2000). The dyads and triads formed in *B. brizantha* were produced by failure in cytokinesis, these microspores developed into 2 N pollen through reinstitution of nucleus. It is possible that a similar process occurs in PI 399073 as this type of pollen was observed in anthers of this landrace. This type of meiotic behavior could limit the breeding potential of a particular genotype if the progeny exhibits these meiotic abnormalities. Precocious pollen germination in soybean genotypes was previously described by Kaur et al. (2005) as a strategy that might facilitate a high degree of selfing and interfere in hybridization efforts. This could explain the production of pods from partially open flowers observed in the cultivar Williams.

These findings are limited to the heterozygous plants that were evaluated in this study, thus the mechanism behind the meiotic abnormalities in PI

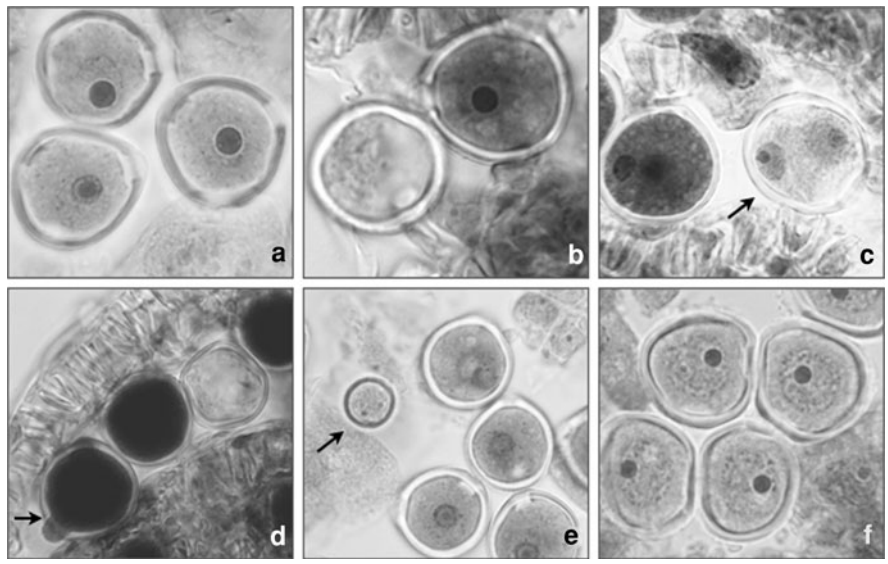


Fig. 4 Microspores characteristics among parental genotypes and one RIL: **a** PI 408211B, mononucleated grains; **b** PI 399073, sterile grain; **c** Williams, binucleated grain; **d** Williams, grain germinating inside the anther; **e** Line 1403 ($BC_4F_{2:3}$), microcyte grain and **f** Line 37 ($F_{4:5}$), mononucleated grains

399073 and its progeny, and what role if any these abnormalities play specifically in the high degree of segregation distortion at the *Rps*8 locus still needs to be explored. In this study, the meiotically abnormal lines originated from a cross where PI 399073 was used as pollen donor, whereas in the meiotically normal lines PI 399073 was the pollen recipient; it is unknown if abnormalities in megasporogenesis are occurring, if the ovules were affected, this could explain why the differences were found between these two populations. Unfortunately lines from reciprocal crosses were not available at the time of this study. In the future, if these lines are available they can be used to determine if the meiotic abnormalities depend on the genotype used as donor parent or on the geographic/genetic distance between the parents. Future studies will focus on the *Rps*8 region and its association with the chromosome fragments, laggards, micronuclei and microcytes. This will only be possible if BAC clones from PI 399073 and Williams 82 located in this region are identified and fully characterized.

Acknowledgments This project was supported by State and Federal Funds appropriated to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University. Funding was also provided in part through soybean check off dollars from Ohio Soybean Council, Iowa Soybean Association, and United Soybean Board. We thank Steven St. Martin and Ron Fioritto for making the crosses and developing populations that generated the RILs evaluated on this study, Sue Ann Berry for the phenotypic evaluation of the RILs, and Dr. Tea Meulia, at the Molecular and Cellular Imaging Center (MCIC) at OARDC, for assistance with microscopy imaging. We also thank Dr. Randy Shoemaker, Dr. Saghai Maroof and Dr. Steven St. Martin for critical discussions throughout this course of study.

References

Adamowski EV, Pagliarini MS, Batista LAR (1998) Chromosome elimination in *Paspalum subciliatum* (notata group). Sex Plant Reprod 11:272–276

Albertsen MC, Palmer RG (1979) A comparative light-and electron-microscopic study of microsporogenesis in male sterile (*ms*1) and male fertile soybeans (*Glycine max* [L.] Merr.). Am J Bot 66:253–265

Baptista-Giacomelli FR, Pagliarini MS, Almeida JL (2000) Elimination of micronuclei from microspores in a Brazilian oat (*Avena sativa* L.) variety. Genet Mol Biol 23:681–684

Bennett MD, Finch RA, Barclay IR (1976) The time rate and mechanism of chromosome elimination in hordeum hybrids. Chromosoma 54:175–200

Bione NCP, Pagliarini MS, Toledo JFF (2000) Meiotic behavior of several Brazilian soybean varieties. Genet Mol Biol 23:623–631

Bione NCP, Pagliarini MS, Almeida LA (2002) An original mutation in soybean (*Glycine max* (L.) Merrill) involving degeneration of the generative cell and causing male sterility. Genome 45:1257–1261

Bione NCP, Pagliarini MS, Almeida LA (2003) Further cytological characteristics of a male-sterile mutant in soybean [*Glycine max* (L.) Merrill] affecting cytokinesis and microspore development. Plant Breed 122:244–247

Bione NCP, Pagliarini MS, Almeida LA (2005) A male-sterile mutation in soybean (*Glycine max*) affecting chromosome arrangement in metaphase plate and cytokinesis. Biocell 29:177–181

Cregan PB, Jarvik T, Bush AL, Shoemaker RC, Lark KG, Kahler AL, Kaya N, VanToai TT, Lohnes DG, Chung J, Specht JE (1999) An integrated genetic linkage map of the soybean genome. Crop Sci 39:1464–1490

Dorrance AE, Schmitthenner AF (2000) New sources of resistance to *Phytophthora sojae* in the soybean plant introductions. Plant Dis 84:1303–1308

Gordon SG, St. Martin SK, Dorrance AE (2006) *Rps*8 maps to a resistance gene rich region on soybean molecular linkage group F. Crop Sci 46:168–173

Grau CR, Dorrance AE, Bond J, Russin J (2004) Fungal diseases. Soybeans: improvement, production, and uses. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, pp 679–763

Gwata ET, Wofford DS, Pfahler PL, Boote KJ (2003) Pollen morphology and in vitro germination characteristics of nodulating and nonnodulating soybean (*Glycine max* L.) genotypes. Theor Appl Genet 106:837–839

Hymowitz T (2004) Speciation and cytogenetics. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production, and uses. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, pp 97–136

Jin W, Palmer RG, Horner HT, Shoemaker RC (1999) *Fr*1 (root fluorescence) locus is located in a segregation distortion region on linkage group K on soybean genetic map. J Hered 90:553–556

Kaur S, Nayyar H, Bhanwra RK, Kumar S (2005) Precocious germination of pollen grains in anthers of soybean (*Glycine max* (L.) Merr.). Soybean Genet Newslet 32:1–10

Keim P, Olson TC, Shoemaker RC (1988) A rapid protocol for isolating soybean DNA. Soybean Genet Newslet 15: 150–152

Kollipara KP, Singh RJ, Hymowitz T (1995) Genomic relationships in the genus *Glycine* (Fabaceae: Phaseoleae): use of a monoclonal antibody to the soybean Bowman-Birk inhibitor as a genome marker. Am J Bot 82:1104–1111

Koti S, Reddy KR, Reddy VR, Kakani VG, Zhao D (2005) Interactive effects of carbon dioxide, temperature, and ultraviolet-b radiation on soybean (*Glycine max* L. Merr) flower and pollen morphology, pollen production, germination, and tube lengths. J Exp Bot 56:725–736

Kumar G, Rai P (2006) Partial genome elimination through micronuclei in soybean (*Glycine max*). Nat Acad Sci Lett 29:417–421

Kyle DE, Nickell CD, Nelson RL, Pedersen WL (1998) Response of soybean accessions from provinces in southern China to *Phytophthora sojae*. Plant Dis 82:55–59

Leppik EE (1970) Gene center of plants as sources of disease resistance. Annu Rev Phytopathol 8:323–344

Lohnes DG, Nickell CD, Schmitthenner AF (1996) Origin of soybean alleles for Phytophthora resistance in China. Crop Sci 36:1689–1692

Lorieux M, Goffinet B, Perrier X, Gonzalez de Leon D, Lanaud C (1995a) Maximum-likelihood models for mapping genetic markers showing segregation distortion. 1. Backcross populations. Theor Appl Genet 90:73–80

Lorieux M, Perrier X, Goffinet B, Lanaud C, Gonzalez de Leon D (1995b) Maximum-likelihood models for mapping genetic markers showing segregation distortion. 2. F2 populations. Theor Appl Genet 90:882–889

Lu C, Zou J, Ikehashi H (2000) Gamete abortion locus detected by segregation distortion of isozyme locus *Pgi*1 in indica-japonica hybrids of rice (*Oryza sativa* L.). Breed Sci 50:235–240

Lu H, Romero-Severson J, Bernardo R (2002) Chromosomal regions associated with segregation distortion in maize. Theor Appl Genet 105:622–628

Lyttle TW (1991) Segregation distorters. Annu Rev Genet 25:511–581

Mahama AA, Deaderick LM, Sadanaga K, Newhouse KE, Palmer RG (1999) Cytogenetic analysis of translocations in soybean. J Hered 90:648–653

Matsushita S, Iseki T, Fukuta Y, Araki E, Kobayashi S, Osaki M, Yamagishi M (2003) Characterization of segregation distortion on chromosome 3 induced in wide hybridization between indica and japonica type rice varieties. Euphytica 134:27–32

McCormick S (1993) Male gametophyte development. Plant Cell 5:1265–1275

Ortega MA, Tucker DM, Peiffer G, Pipatpongpinyo W, Berry SA, Hyten DL, Cregan P, Shoemaker R, St. Martin SK, Saghai-Maroof MA, Dorrance AE (2008) Development of molecular markers for fine mapping of the *Rps*8 gene locus in soybean. Abstract. Phytopathology 98:S117

Ortega MA, Tucker DM, Berry SA, St. Martin SK, Saghai-Maroof MA, Cregan PB, Hyten DL, Shoemaker RC, Dorrance AE (2010) Is *Rps*8 alone? Evidence for different genes for resistance to *Phytophthora sojae* on the chromosome 13 of soybean Pl 399073. Phytopathology 100:S189

Palmer RG, Sun H, Zhao LM (2000) Genetics and cytology of chromosome inversions in soybean germplasm. Crop Sci 40:683–687

Rattenbury JA (1956) A rapid method for permanent acetocarmine squash preparations. Nature 177:1185–1186

Risso-Pascotto C, Pagliarini MS, Borges do Valle C, Mendes-Bonato AB (2003) Chromosome number and microsporogenesis in a pentaploid accession of *Brachiaria brizantha* (Gramineae). Plant Breed 122:136–140

Schreiber J (1954) Staining plant and animal chromosomes by the feulgen-acetocarmine sequence. Biotech Histochem 29:285–291

Singh RJ (1993) Plant cytogenetics. CRC, Boca Raton

Subrahmanyam NC, Kasha KJ (1973) Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. Chromosoma 42:111–125

Thomas HM (1988) Chromosome elimination and chromosome pairing in tetraploid hybrids of *Hordeum vulgare* × *H. bulbosum*. Theor Appl Genet 76:118–124

Wrather JA, Koenning SR (2006) Estimates of disease effects on soybean yields in the United States 2003 to 2005. J of Nematol 38:173–180

Wrather JA, Stienstra WC, Koenning SR (2001) Soybean disease loss estimates for the United States from 1996 to 1998. Can J Plant Pathol 23:122–131

Yamanaka N, Ninomiya S, Hoshi M, Tsubokura Y, Yano M, Nagamura Y, Sasaki T, Harada K (2001) An informative linkage map of soybean reveals QTLs for flowering time, leaflet morphology and regions of segregation distortion. DNA Res 8:61–72